Detection and identification of antibiotic-resistant bacteria in water samples

Letícia Correia Fortes
Mestrado em Genética Forense
Departamento de Biologia
2016

Orientador
Manuela Oliveira, Ph.D.
Instituto de Patologia e Imunologia Molecular da Universidade do Porto
Faculdade de Ciências da Universidade do Porto

Coorientador
Luísa Azevedo, Ph.D.
Instituto de Patologia e Imunologia Molecular da Universidade do Porto
Faculdade de Ciências da Universidade do Porto

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Acknowledgments

First and foremost, I would like to thank my parents for their extraordinary support in my personal and academic life. I am very grateful for the education that they transmitted to my brothers and me. I thank my family for encouraging me and believe in me when I was discouraged.

Secondly, I would like to express my sincere gratitude to my academic supervisor Manuela Oliveira for her extraordinary support of my work and thesis. I thank her for sharing her knowledge and always encouraging me to stay motivated. Thank you for your comprehension in difficult moments of “white gel”. I cannot forget my co-supervisor Luisa Azevedo for her extraordinary support of the internship and the resolution of initial problems. Thanks to both for all.

Furthermore, I would like to express my gratefulness to Isabel Cristina Rosas and Patricia Costa from SAGILAB – Laboratório de Análises Técnicas, SA for the collaboration in this work, providing the indispensable water samples. Also, my thanks to the personnel from the Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, namely Michaela Lackner, Thomas Larentis, Michael Berktold, and Rita Caramalho for their extraordinary help in obtaining some of the results and the special thank you for the important isolates provided to perform this study. Thanks for your help from Austria, which was crucial for the completion of this thesis. I also acknowledge Luísa Peixe and Carla Oliveira, from the Faculty of Pharmacy from the University of Porto, for providing the reference strains used in the present work.

Now, I would like to thank my friends, these wonderful people who crossed in my life, Cátia, Sara, Eduarda, Ana, Fábio and Claúdia. Thanks for your patience and your support during all my academic life. I would like to thank especially, for encouraging me during all my master’s degree, with several ups and downs. Thank you to all my friends and family for the support given to me during this two difficult years.

For last I would like to express the importance of the music to me, and its help in difficult moments. I would like to thank all the people that enable the presence of the music in my life always. The music also helped me a lot during this step.

Abstract

The discovery of antibiotics constituted a breakthrough for medicine, making possible to combat microbial infections, which until then were uncontrollably decimating people. However, as humans adapt to changes, bacteria also possess this ability. Bacteria rapidly adapted to the rise of the antibiotic era, developing mechanisms of defense against them. Horizontal gene transfer (HGT) appeared as a mechanism that allowed the dissemination of the resistance between bacteria.

The resistance acquired by the bacteria is a parallel phenomenon that accompanies the rising and use of antibiotics, initially was detected in hospitals and then identified in the environment, becoming a public health concern.

The increase of resistant bacteria in environmental waters has been a matter of concern in several research works. The persistence resistant bacteria after water treatments with antibiotics and disinfectants alerted to the spreading of resistant bacteria in the environment.

In this thesis, bacteria isolated water samples were studied, with the aim of detecting and identifying resistant bacteria in water samples, along with discovering to which antibiotic the bacteria are resistance/susceptible. To achieve this aim, samples were identified using molecular methods, such as 16S sequences and MALDI-TOF MS. Three different genera were identified, being *Staphylococcus* the most abundant bacteria present. The next step was to study several genes associated with resistances (*aacA-aphD, mecA, qacC, vanA, vanB e vanC*), and subject the isolates to an antibiogram. From all the studied isolates, some displayed resistance to several antibiotics, while others were widely susceptible to these drugs, with a higher number of bacteria being resistant to fusidic acid.

The continuous screening of the microbial population from water samples presents a significant aspect under the scope of Forensic Sciences. A deeper knowledge of such communities will help to detect and prevent possible outbreaks of pathogenic bacteria as well as to map and control the spread of antibiotic resistances into the environment that ultimately are transmitted to humans causing a wide range of public health problems. In the present study, the presence of antibiotic-resistant bacteria in water samples will be studied.

Keywords

Antibiotic; antibiotic-resistance; Bacteria; resistant-bacteria; water.

Resumo

A descoberta dos antibióticos constituiu um grande avanço para a medicina, tornando possível combater infeções microbianas, que até então, dizimavam populações. Contudo, da mesma forma que os humanos são capazes de se adaptar à mudança, também as bactérias possuem esta capacidade. As bactérias adaptaram-se rapidamente ao aparecimento dos antibióticos desenvolvendo mecanismos de defesa. A transferência horizontal de genes aparece como um dos mecanismos que permitiu a disseminação das resistências entre bactérias.

A resistência a antibióticos foi inicialmente detetada e identificada em hospitais, ao longo dos anos verificou-se o seu aparecimento no meio ambiente tornando-se, assim, uma preocupação em termos de saúde pública.

O aparecimento de bactérias resistentes em águas tem sido uma preocupação constante para os investigadores. A persistência destas bactérias, após tratamento das águas com antibióticos e desinfetantes, alerta para a propagação das bactérias resistentes no ambiente.

Neste trabalho foram estudadas isolados bacterianas isoladas a partir de amostras de água com o objetivo de detetar e identificar as bactérias existentes, assim como as resistências associadas. Foram utilizados métodos moleculares de identificação - 16S e MALDI-TOF MS. Foram encontradas três géneros diferentes de bactérias sendo *Staphylococcus* o mais abundante. Vários genes associados a resistências foram estudados (*aacA-aphD, mecA, qacC, vanA, vanB e vanC*), e os isolados foram sujeitos a um antibiograma. Das amostras estudadas, algumas apresentaram resistência a vários antibióticos, por outro lado, outras apresentam-se amplamente suscetíveis.

O estudo contínuo das populações microbianas é um procedimento importante para rastrear as fontes de surtos de patogénicos. Através de ferramentas da genética forense, como a identificação molecular, é possível controlar a propagação de microorganismos resistentes a antibióticos. Além disso, permite o conhecimento das populações microbianas, especialmente as patogénicas, ajudando na produção de novos antibióticos. Esta tese centra-se na identificação de bactérias resistentes ocorrentes em meios aquáticos.

Palavras-chave

Bactérias; antibióticos; resistência a antibióticos; bactérias resistentes; ambientes aquáticos.

Table of contents

Figures Index

Tables Index

Abbreviations list

AMR - Antimicrobial resistance

AR – Antibiotic resistance

BLASTN – Basic local alignment search tool nucleotide

DNA - Deoxyribonucleic acid

EARS-Net – European antimicrobial resistance surveillance network

ECDC - European Centre for Disease Prevention and Control

FFUP – Faculty of Pharmacy, University of Porto

HGT - Horizontal gene transfer

IMU – Innsbruck Medical University

MALDI-TOF MS – Matrix-assisted laser desorption/ionization mass spectrometry

MEGA – Molecular Evolutionary Genetics Analysis

MGE – Mobile genetic elements

MRSA – Methicillin-resistant *Staphylococcus aureus*

NCBI – National Center for Biotechnology Information

PCR – Polymerase chain reaction

rRNA – Ribosomal ribonucleic acid

VRE – Vancomycin-resistant Enterococcus

WHO - World Health Organization

1. Introduction

1.1 Antibiotic resistance

Throughout the years, there has been a major concern in controlling pathogenic bacteria that affect the organism's homeostasis. Therefore, several works focused its attention on the production the most adequate antibiotics for a given target microbe [1].

Antimicrobial compounds, natural or synthetic , have a low molecular weight and interfere with the natural functions of bacteria, inhibiting (bacteriostatic) or killing (bactericidal) the microorganism, mostly involving cell-to-cell signalizing networks [2]. In the late 1930's antibiotics were developed being classified according to their origin. Natural antibiotics are produced by microorganisms (bacteria and fungi), such as penicillin; semisynthetic antibiotics are those obtained from the core of penicillin, such as methicillin; and the synthetic antibiotics, are those obtained chemically, such as sulfonamides. Additionally, antibiotics can also be classified based on their biological effect: bactericidal agents, which kill bacteria, and bacteriostatic agents, which limit bacterial growth [3]. Usually, the antibiotics are mentioned through different classes such β-lactams, fluoroquinolones, sulfonamide, tetracycline, among others [4].

According to Nathan and Cars [5], modern medicine covers not only the treatment of severe infections but also the protection against infections acquired during surgical procedures. Therefore, antibiotics are considered one of the major discoveries of modern medicine and the most successful forms of therapy, alleviating human morbidity and mortality [1, 6, 7]. Moreover, antibiotics provided an outstanding contribution to the increase of the lifespan by changing the outcome of several bacterial infections - both acquired in the health care centers (nosocomial infections) or outside theses settings (community-acquired). As such, these medicines play a pivotal role in the success of some advanced medical practices [1].

The literature describes the "golden era" of antibiotic discovery from 1930's to 1960's. During this period, the pharmaceutical industry observed an exceptional advance in the production ofnew antibiotics [5]. However, its efficiency was compromised by the rising and rapid spreading of antibiotic resistance in bacteria [8, 9]. The high consumption and abuse of antibiotics in human and veterinary medicine are possible explanations for the worldwide growing phenomenon of antimicrobial resistance (AMR) [3]. As such, with the aim of controlling this emergence of resistances, several agencies and governmental reports, have emphasized the importance of reducing antimicrobial prescription and consumption [10]. According to European Centre for Disease Prevention and Control (ECDC) [11] "AMR is a microorganism's ability to resist the

action of one or more antimicrobial agents". AMR can raise severe consequences since treatment with specific antibiotics is one of the most efficient methods to treat serious infections [11].

All organisms present an exceptional ability to adapt to changes and microorganisms are not an exception. Throughout evolution, microorganisms have demonstrated great adaptability, surviving in different conditions, such as frozen or boiling waters, extreme pHs, and elevated pressure environments. These microorganisms present high population numbers, a remarkable genomic elasticity, and a great capacity to exchange genetic information among different species [12]. Consequently, it is not without surprise that, over the last years, bacteria have developed a vast diversity of metabolic and protective mechanisms and have become resistant to various antimicrobial agents [3, 13].

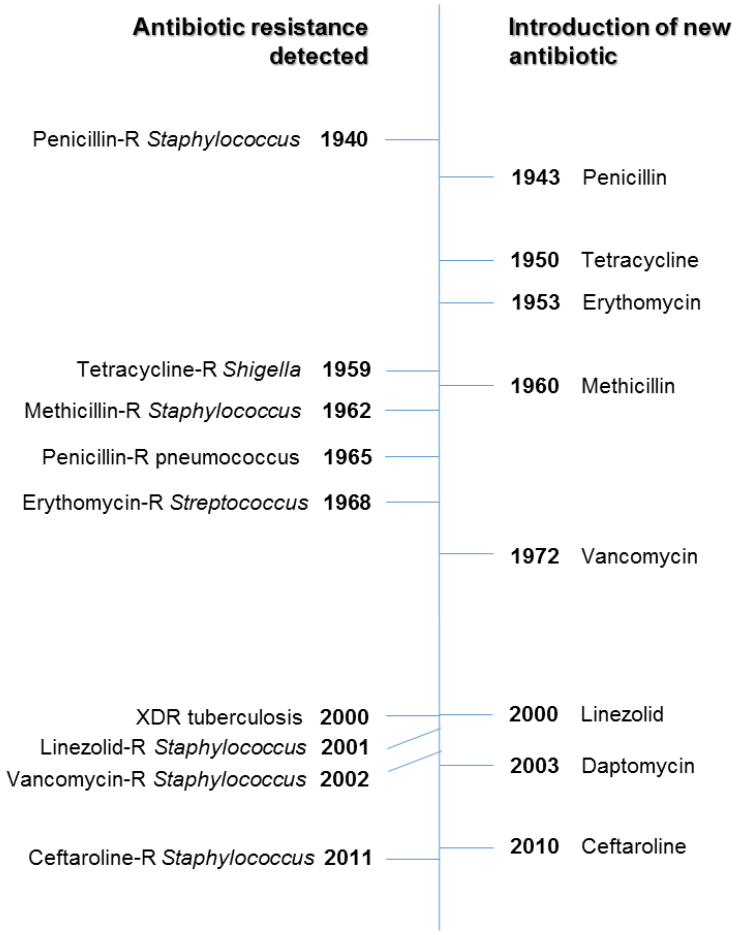


Figure 1.1.1 - Adapted timeline of an antibiotic introduction and developing antibiotic resistance in population adapted from Ventola [14].

Antimicrobial resistance is not a recent phenomenon. However, the frequency of AMR in many bacterial pathogens is increasing promptly and remains a severe global health problem, with the need for new solutions (figure 1.1.1) [15]. In Europe, in the year 2009, analyses from ECDC estimated that about 25000 deaths, were a result of infections caused by resistant bacteria [11].

The development of antibiotic resistance is a natural process providing bacteria the remarkable ability to become resistant, through enrichment of resistance mutations or the transference of resistant genes between bacteria [11]. For the pharmaceutical industry, this phenomenon brings some problems. The rise (sooner or later) of antibiotic resistance and the development of resistance to newly developed molecules, hampers the production of effective antibiotics [3]. Moreover, the main challenge for the pharmaceutical industry relies on the identification of the events that result in bacterial resistance, from the gene responsible for resistance to the downstream effects. Bacterial resistance expression is usually controlled by transcriptional regulators that can repress or activate the transcription of the referred gene [8].

The World Health Organization (WHO) and European Antimicrobial Resistance Surveillance Network (EARS-Net) have recognized the importance of monitoring antimicrobial drug resistance in bacterial pathogens, to offer better treatments for microbial infection [16]. The knowledge of the antimicrobial microorganism susceptibility profiles can also be a significant tool for medicine and along the years the WHO passed down to microbiologists the responsibility of providing periodic reports on antimicrobial susceptibility patterns with the aim of monitoring resistance patterns [17].

1.1.1 Antimicrobial resistance mechanisms

Antibiotic's target can be a crucial enzyme, protein synthesis or others cellular process, cell wall biosynthesis, DNA transcription or replication [18]. Nowadays, antibiotic drugs face the problem of having a limited clinical efficacy, mainly due to the emergence of resistant pathogens, minimizing their effective treatment of bacterial infections [6, 19]. Most antibiotics are originated in nature and synthesized by different microorganisms. Therefore, many bacteria have been exposed to antibiotics for a long time. Consequently, bacteria (producers and bystanders) developed several mechanisms to resist the action of antibiotics, conferring antibiotic resistance [3, 18]. So, when a pathogen population is subjected to a certain antibiotic, the pre-existing resistant strains will be selected (selective pressure), being able to survive and multiply a resistant progeny, that will replace the original non-resistant community [3, 12].

The selection induced by the presence of an antibiotic also plays a role in selecting bacteria with a high rate of mutation, termed the hypermutators or mutators. The presence of such alleles increases the possibility of beneficial mutations for the bacteria, leading to an acceleration of the evolutionary rate under certain conditions [12]. Resistance to penicillin and streptomycin was quickly reported after their discovery and was one of the first resistance to antibiotics reported [6].

Important functions of bacteria, such cell wall and protein synthesis, are targets for antibiotics, so the resistant mutants have fitness changes. The mutational change can affect the bacteria's growth, persistence, and survival, so it is said that the mutation has a fitness effect/cost (capacity of survival). The mutation can have a fitness cost in bacteria that are in environments in the absence of antibiotic, meaning that some resistances make bacteria more susceptible when in an antibiotic free environment. However, the decreased fitness may be counterbalanced by compensatory mutations without the loss of the resistance. The compensatory mutation associated with mutations with a deleterious effect reduce or eliminate the fitness cost of mutation (compensatory evolution). So, the fitness cost can be minimized through these deleterious mutations, complementing the resistance mutation, ensuring the prevalence of bacteria [18, 20, 21].

Antimicrobial resistance mechanisms have been studied and associated with genetic and biochemistry aspects of different bacterial cell function [9]. The mechanisms conferring antimicrobial resistance include membrane permeability alterations (exclusion of the antibiotic by the cell membrane or intracellular sequestration; e.g.:

Gram-negative bacteria confer impermeability to hydrophobic compounds), multidrug efflux systems (pump the antibiotic out of the cell; e.g.: aminoglycoside resistance), antimicrobial target alteration (modification of intracellular receptor), enzymatic degradation and/or deactivation of the antimicrobial drugs (resistance to β-lactams antibiotics), and new metabolic ways (synthesis of altered enzymes) [3, 8, 18, 22].

In the mid-1950, the resistance transference was discovered, leading to a better knowledge of antibiotic resistance gene spreading, implying the existence of a large reservoir of antibiotic resistance genes circulating horizontally and vertically along the microbial populations (for example, phylogenetic analyses of different proteins associated to β-lactamases suggest an ancestor) [6]. Therefore, antibiotic resistance is usually considered a characteristic acquired for previously susceptible bacteria that may be involved in the horizontal gene transfer (HGT), or the occurrence of mutations in chromosomally located genes that are vertically transmitted to a new generation bacteria [19]. Besides mutations, other mechanisms can originate genetic variations in bacteria, such as the intra-genomic reorganization of genomic sequences (intra-chromosomal recombination). These mechanisms are pivotal in the evolution and adaptation of bacteria, along with the evasion of the immune response and the distribution of genes responsible for virulence increase and/or antibiotics resistance. Most antibiotics resistance has been acquired by lateral transfer of resistance genes between bacteria within the communities [12].

The results from cloning and sequencing genes related to antibiotic resistance revealed multiple genes acting as genetic sources of resistance, often transferred between microbial communities by horizontal and vertical transfer events [6]. Moreover, recent works demonstrated that transfer events constitute the major sources of evolution, deeply influencing bacterial survival [12].

1.1.1.1 Intrinsic and acquired resistance

Bacterial resistance to antibiotics can be intrinsic or acquired. Intrinsic resistance refers to the existence of genes that generate a resistant phenotype, an innate characteristic of a given species and universally found within the genome of bacterial species, frequently encoded in the chromosome and is independent of antibiotic selective pressure and gene transference [9, 19]. Bacterial genome sequencing has revealed several putative intrinsic gene functions in bacteria that can lead to resistance [9]. Some microorganisms have intrinsic resistance mechanisms encoded in their resistome (set of antibiotic resistance genes) expressed at a basal level and conferring naturally reduced susceptibility to antibiotics [23]. In certain of these intrinsically resistant microorganisms, the use or misuse of antibiotic does not affect the level or mechanism of resistance [3]. Some bacterial species occurring in habitats of low antibiotic pressure can also present intrinsic resistance of an antimicrobial compound [24].

Acquired antibiotic resistance is not part of the natural characteristics of a bacteria, being resistance genes obtained by the transfer of genetic information induced by selective pressure[24].

Through the years, several possible pathways of resistance acquisition from microorganisms have been studied, with an emphasis on the process of gene transference between microorganisms. The horizontal gene transfer (HGT) gained relevance with the discovered of the presence of putative bacterial gene sequences in eukaryotic genomes. This process has become a turning point in genome evolution and antibiotic resistance acquisition in microorganism [9]. Although mutations are infrequent, they constitute a way to acquired resistance through genetic changes [3].

1.1.1.1.1 Horizontal gene transfer

Bacteria possess the ability to interchange genes amongst species, a phenomenon known as horizontal gene transfer (HGT). This transfer constitutes an an important event in human pathogens' evolution and a driving force in shaping the prokaryotic genomes. Additionally, this is an important mechanism of adaptation and evolution in bacteria [19, 24-26]. In HGT events, a mobile segment of DNA is transferred from one part of the genome to another or even between genomes - among prokaryotes, from Bacteria to Eukaryotes, from Bacteria to Archaea, and from animals to Bacteria [23, 27, 28]. However, HGT is more frequent among phylogenetically closely bacteria due to the similarities in the sequences of DNA and proteins, and common occurrence in the same ecological niches [29]. HGT conjugation allows the acquisition of certain genetic information for the use of new substrates and survival in toxic environments [26]. Also, through HGT bacteria can acquire genes for antibiotic resistance, resulting in new virulence factors and alternative metabolic pathways, contributing to the emergence of novel "superbugs" [26, 30, 31]. Aspects such as the close associations between organisms in nature, high natural selection (hospitals, bioremediation spots, high biodiversity in natural habitats), large concentrations of viral DNA in the ocean, abundance of DNA in soil, the natural competition between microorganisms, among others, can contribute to the HGT process [32].

Mobile genetic elements (MGEs) have an important role in the adaptation process of bacteria and provide a way for the transfer of genetic information in the HGT process, between and within bacterial species. MGEs include gene cassettes, transposons, integrons, plasmids, bacteriophages, nanotubes, and membrane vesicles, and present inter and intracellular mobility [28, 29]. However, each of these mobile elements exhibits its individual characteristics, for example, gene cassettes, transposons, and plasmids present a very broad host range, while bacteriophages have a reduced host range [28, 33]. A significant number of plasmids stimulates a conjugation transfer of bacterial DNA to cells in several organisms; distinct bacteria, yeasts, fungi, and plants mobilize operons and regulons [34]. As for instance, plasmids are associated with the degradation of aromatic compounds and the virulence determinants of *Yersinia.* Bacteriophage elements present a narrow host range, allowing the mobility of regulators and also constituting virulence factors [28]. Through a single transfer event, bacteria can acquire a high level of resistance to a given antibiotic and simultaneously became resistant to several antibiotics. The integrons are the main genomic fragments responsible for these acquisitions since they transport and express several resistance

genes. When associated with a plasmid, integrons have the ability to turn a susceptible bacteria into a multidrug-resistant bacteria [18].

The genetic transfer occurs by three main mechanisms: transformation (uptake of free DNA from the environment), conjugation (plasmid-mediated transfer), and transduction (bacterial virus-mediated transfer), including the mobile elements previously described [30, 35].

The transformation mechanism consists of bacteria acquiring a free DNA from the environment. This mechanism enables bacteria to obtain material for DNA repair and nutrition but also permits the acquisition of new genetic components that help the adaptation to different environmental conditions [36]. Natural transformation is a complex process of DNA transport across bacterial membranes that involves the DNA uptake, recombination of homologous DNA or reconstruction of a plasmid, and the phenotypic expression [36].

The conjugation mechanism is used by bacteria for the transfer of an extensive variety of genes [37]. As natural transformation, conjugation is mediated by semi-autonomous vectors, such as plasmids [38]. The conjugation mechanism occurs when bacteria directly transfer the genes to another cell through a tube-like structure, known as the pilus, which helps the transference of the plasmids, genomic islands and integrative and conjugative elements [23, 37]. This mechanism has been studied in several bacteria. For instance, it has been demonstrated the plasmid transfer between *Escherichia coli* and cyanobacteria, and between *E. coli* and *Saccharomyces cerevisiae* [23, 30].

The transduction mechanism is mediated by bacteriophages (bacterial viruses) that transfer genes from one bacteria to another, without being necessary to stablish a physical contact between the cells involved [30, 39]. Several studies reported that bacteriophages transport many functional bacterial genes, serving as a reservoir for genes in different environments and acting as MGE in the genetic transfer between bacteria [40]. Moreover, they have an important role in the transfer of antibiotic resistance genes amongst bacteria. Also, resistant genes in phage particles have been detected in nature [40].

Therefore, the HGT mechanisms, and particularly the mobile genetic elements from the bacteria involved in this process, presents a high interest for practical applications in genetics and biotechnology[28].

1.2 Occurrence of antibiotic resistance in the water environments

Since its discovery, the production of antibiotics has continually increased. Moreover, its consumption by humans or animals and utilization for agricultural purposes has been augmented, discharging significant quantities of antibiotics into the environment [41, 42]. This significant increase has led some authors to classify these pharmaceuticals as a class of micro-contaminants at the end of the 20th century [43]. Two source of pharmaceuticals pollution can be identified - point source pollution and diffuse pollution. As such, industrial and hospital effluents are regarded as point source pollution, while agricultural and urban overspills are some examples of diffuse pollution, usually presenting lower antibiotic concentrations when compared to point pollution [4, 41]. The principal sources of antibiotic released to the environmental are wastewaters from the process of antibiotics production (industrial effluent), hospitals, the inappropriate discard of unused and life expired antibiotics, and the excretion from patients (effluents from urban wastewater) [4, 41-43].

In the last decades, an outstanding number of works on antibiotic resistances has been published, reflecting the increasing interest from the scientific community and the international organizations about this topic, with relevant initiatives such the projects like PHARMAS, and the networking COST Action DARE [42].

The wastewater biological treatment process offers a suitable environment for the development of resistance due to the presence of sub-inhibitory concentrations of antibiotics [42]. Consequently, the process makes it easy for bacteria to acquire resistance and enables the transference of antibiotic resistance genes [41, 42]. These facts concern the scientific community since several of these genes are found in integrons, transposons or plasmid, enabling HGT between bacteria [44]. Furthermore, the high concentrations of antibiotics in a wastewater treatment, such as the activated sludge processes, compromised the biological approach. Antibiotics inhibit bacterial growth and functions, such as the removal of organic nutrients [41]. Moreover, some studies revealed that the antibiotics removal is incomplete in sewage treatment plants, being the concentration of antibiotics in effluent similar to that of the influent [4].

Molecular biology methods have been used over the years to examine water quality. These methods enable the detection of microorganisms present in the water and its clinical relevance. Many works performed along the water cycle have demonstrated the occurrence of clinically relevant antibiotic resistance genes(Table 1.2.1) [42]. Some

resistances are associated to urban wastewaters, such the ampicillin resistance gene *ampC*. However, the vancomycin resistance gene and the appearance of a multiresistant *Pseudomonas aeruginosa* are linked to hospital wastewaters. These microorganisms are the most frequently found in hospital wastewaters comparing to effluents from housing areas, indicating that hospitals can be a hotspot for the spread of clinically important antibiotic-resistant bacteria [42].

Table 1.2.1 - Clinically relevant antibiotic resistance genes present in aquatic environments.

Type of resistance	Resistance gene	Microorganism	Ref.
β-lactam	*ampC*	*Enterobacteriaceae*	[42]
methicillin	*mecA*	*Staphylococci*	*[42]*
carbapenem	*blaVIM*	*Pseudomonas aeruginosa*	*[42]*
vancomycin	*vanA*	*Enterococci*	*[42]*
tetracycline	*Tet(M)*	*Enterococcus faecalis*	*[45]*

Several microorganisms have been investigated due to their clinical relevance, such as Enterococci, *E. coli*, Staphylococci, among others [42]. A study performed in Portugal, in sewage water treatment plants, observed the presence of numerous multiresistant Enterococci in urban sewage. Moreover, it was also concluded that the hospital's sewage affect the quantities of resistant microorganism in wastewater. The bacteria are multiresistant when they resist at least three families of antibiotics.[46].

Swimming pools, another aquatic environment, have been identified as potential source of resistances. These environments represent some public health risks to bathers due to bacterial or chemical contamination. As such, *Staphylococcus aureus* is frequently used as an indicator of the hygienic quality of swimming pools due to its higher resistance to chlorine and other halogen disinfectants, when compared to coliforms and enterococci. Also, *S. aureus* has been described as a possible causative agent for the occurrence of skin, eye, and urinary infection in swimming pools users [47]. In Greece, it was reported that multiresistant strains were isolated from swimming pool waters, being *S. aureus* the most frequent bacteria found [47].

2. Material and Methods

2.1 Strains and DNA extraction

Bacterial isolates (001 to 107) obtained from water samples were kindly provided by SAGILAB. Samples were frozen in LB medium with 20% glycerol until further use (Appendix 1).

The strains used as positive and negative controls, the ATCC 29213 *S. aureus* strain and the VRE - Vancomycin-resistant *Enterococcus*, were kindly provided by Professor Doctor Luísa Peixe from Faculty of Pharmacy, University of Porto (FFUP). The MRSA - methicillin-resistant *S. aureus* and the multiresistant *S. aureus* strains (M110/16 to M120/16) were kindly provided by Doctor Michael Berktold from Division for Hygiene and Medical Microbiology of Innsbruck Medical University (IMU) (Appendix 1).

Colony-PCR was performed as previously described by Woodman (2008) with some modifications. Briefly, each colony was harvested using a toothpick to a tube containing LB medium with 20% glycerol and heated at 95ºC, for 10 min, to ensure cell lysis. The cell lysate was then used for gene amplification.

2.2 Gene amplification and sequencing

The primers used in this study were for bacteria identification - 16S universal primers, for the specific identification of *Staphylococcus aureus* - Sa primers, and for the molecular detection of resistance - aacA-aphD, mecA, vanA, vanB, vanC, and qacC (Table 2.2.1). All the primers were purchased from SIGMA (USA).

The PCR was performed using a mixture containing 1µL of the primer reverse (2µM), 1µL of the primer forward (2µM), 5µL of the MyTaq DNA polymerase, 0.5µL of H_2O, 0.5µL of Qsolution and 2µL of extracted DNA. The PCR mixtures were subjected to thermal cycling (5min at 95ºC, for initial denaturation; 35 cycles of 30s at 95ºC for denaturation, 30s at variable temperature depending on the set of primers, for annealing, and 1min at 72ºC, for extension; and 5min at 72ºC, for final extension) in an Applied Biosystems GeneAmp PCR System 2700. Annealing temperatures are described in table 2.2.1.

Amplification products were confirmed on polyacrylamide gels and visualized by silver staining as previously described by Qu, Li [48] with some modifications. Briefly, the gels were fixed in ethanol (10%), for 10min, impregnated with nitric acid (1%) and silver nitrate (0.2%), for 20 minutes, developed with a solution of sodium carbonate (3%) and formaldehyde (0.02%), and revelation was stopped with acetic acid (10%).

Table 2.2.1 - List of the primers used in these assays and its characteristics.

Primer pair	Target gene	Phenotype/ resistance	Sequence (5'-3')	Amplicon size (bp)	Annealing extension temperature (ºC)	Ref.
16S 1	*16S*	Bacteria identification	GGA GGA AGG TGG GGA TGA CG	241	60	[49]
16S 2			ATG GTG TGA CGG GCG GTG TG			
Sa 1	*Sa*	*Staphylococcus* identification	AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG	108	60	[50]
Sa 2			CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA			
aacA-aphD 1	*aacA-aphD*	Aminoglycoside resistance	TAA TCC AAG AGC AAT AAG GGC	227	57	[51]
aacA-aphD 2			GCC ACA CTA TCA TAA CCA CTA			
mecA 1	*mecA*	Methicillin resistance	CCT AGT AAA GCT CCG GAA	532	57	[51]
mecA 2			CTA GTC CAT TCG GTC CA			
vanA 1	*vanA*	Vancomycin resistance	ATG CGA ATG TTC CGA AAA TGT	1030	60	[52]
vanA 2			CCC CTT TAA CGC TAA TAC GAT CAA			
vanB 1	*vanB*		GTG ACA AAC CGG AGG CGA GGA	443	60	[52]
vanB 2			CCG CCA TCC TCC TGC AAA AAA			
vanC 1	*vanC*		GAA AGA CAA CAG GAA GAC CGC	796	60	[52]
vanC 2			ATC GCA TCA CAA GCA CCA ATC			
qacC 1	*qacC*	Disinfectant resistance	GGC AAG TCA GGT GAA GAT	249	57	[53]
qacC 2			ATG CGA ATG TTC CGA AAA TGT			

Before sequencing, amplification products were purified with Sephadex. For the sequencing reaction, the solutions were prepared with: 1,5µL of PCR purified amplification product, 1µL water, 2µL of sequencing kit and 0,8µ of primer forward (5µM); and 1,5µL of PCR purified product, 1µL water, 2µL of sequencing kit and 0,8µ of primer reverse (5µM). The sequencing solutions were subjected to thermal cycling (2 min at 96ºC ant then 30 cycles of 15s at 96ºC, 9s at 50ºC and 2min at 60ºC, after all the cycles the reaction ended with 10min at 60ºC) in an Applied Biosystems GeneAmp PCR System 2700. Sequences were amplified, at least once, using both forward and reverse primers. Sequencing data were processed and analyzed with Geneious 5.5.8 and MEGA6 software [54, 55].

2.3 MALDI-TOF MS

The strains were grown in Columbia Blood Agar (Sigma Aldrich) for 48h at 37°C, and further identified using the MALDI Microflex LT (Bruker Daltonics, Bremen, Germany), as previously described [56].

2.4 Antibiogram

The antibiograms were performed using the EUCAST disk diffusion antimicrobial susceptibility testing method, as previously described [57].

3. Results and Discussion

3.1 Sample identification

In this study 30 isolates of bacteria from waters samples, two control strains (ATCC and VRE) and 10 MRSA from a collection of resistant strains were used.

The DNA extracted from the isolates was amplified using 16S primers, universal primers for bacteria as described in previous studies, such as Moreira and coworkers [58] and Martineau and coworkers [49]. All the isolates included in this work showed a positive result for amplification with 16S primers (Table 3.1.1). From all isolates available, 19 randomly selected were sequenced, to identify the strains. Also, in the study by Farkas and coworkers [59], the molecular identification with 16S rRNA gene enabled the identification of *Staphylococcus*, as well as other bacteria, present in water treatment processes. The prevalence of these bacteria in water is a growing concern among researchers, since several studies have been focusing on solving this problem [59]. In the table 3.1.2 results show that six isolates were identified as *S. aureus* (isolates 2, 6, 59, 75, 106, and 107), 11 as *Staphylococcus warneri*, (isolates 7, 16, 33, 60, 68, 69, 74, 84, 85, 91, and 92) and four as *Staphylococcus saprophyticus*, (isolates 97, 98, 104, and 105). The strain ATCC was identified as *S. aureus*, as expected.

Table 3.1.1 - Results of PCR amplification for isolate identification (16S and Sa) and resistances identification (aacA-aphD, mecA, qacC, vanA, vanB and vanC). (+) - positive result; (-) – negative result.[1]

Sample	*16S*	*Sa*	*aacA-aphD*	*mecA*	*qacC*	*vanA*	*vanB*	*vanC*
2	+	+	-	-	-	-	-	-
6	+	+	-	-	-	-	-	-
7	+	+	-	-	-	-	-	-
16	+	+	-	-	-	-	-	-
17	+	-	-	-	-	-	-	-
18	+	-	-	-	-	-	-	-
19	+	+	-	-	-	-	-	-
20	+	+	-	-	-	-	-	-
31	+	-	-	-	-	-	-	-
32	+	+	-	-	-	-	-	-
33	+	-	-	-	-	-	-	-
53	+	+	-	-	-	-	-	-

[1] * The 16S and Sa sequences form these isolates were not amplified since they belong a collection of resistant strain carefully identified and studied.

54	+	+	-	-	-	-	-	-
55	+	+	-	-	-	-	-	-
59	+	+	-	-	-	-	-	-
60	+	-	-	-	-	-	-	-
68	+	+	+	-	-	-	-	-
69	+	+	-	-	-	-	-	-
74	+	+	+	-	-	-	-	-
75	+	+	+	-	-	-	-	-
84	+	+	-	-	-	-	-	-
85	+	+	-	-	-	-	-	-
91	+	-	-	-	-	-	-	-
92	+	-	-	-	-	-	-	-
97	+	-	-	-	-	-	-	-
98	+	-	-	-	-	-	-	-
104	+	+	-	-	-	-	-	-
105	+	+	-	-	-	-	-	-
106	+	+	-	-	-	-	-	-
107	+	+	-	-	-	-	-	-
ATCC	+	+	-	-	-	-	-	-
VRE	+	-	+	-	-	+	-	-
110	*	*	+	+	-	-	-	-
111			+	+	-	-	-	-
112			+	+	-	-	-	-
113			+	+	-	-	-	-
114			+	+	-	-	-	-
115			+	+	-	-	-	-
116			+	+	-	-	-	-
117			+	+	-	-	-	-
119			+	+	-	-	-	-
120			+	+	-	-	-	-

Sequencing was performed for to complete the identification of the isolates. Using the Geneious software sequences were aligned and posteriorly compared with the known sequences in GenBank, using the BLASTN of the NCBI database. The results from BLASTN were transferred to MEGA6 software [54, 55] for phylogenetic reconstruction (Figure 3.1.1). The analysis in MEGA6 showed the proximity between isolates and other strains, such as *S. saprophyticus*, *S. warneri*, *S. aureus,* and others. In the phylogenetic tree were used several strains from Staphylococcaceae (from the isolates included in this study) and one strain of *Pseudomonas aeruginosa* (an outer group for tree rooting). From all the isolates belonging to Staphylococcaceae, 15 nucleotide sequences of isolates showed phylogenetic proximity with *S. warneri*. However, isolates 106, 107, and the strain ATCC presented phylogenetic proximity with *S. aureus*, and isolates 105, 104, 98, and 97 were in the same branch as the *S. saprophyticus*.

DNA extracted from the isolates was amplified with the Sa primers, as previously described by Martineau *et al* [50]. In a previous work performed by Pereira *et al* [60], Sa primers were reported as specific for *S. aureus*, amplifying a 108bp fragment. The results demonstrated that 21 of the 30 isolates amplified the DNA region specific for *S. aureus* (Table 3.1.1), namely isolates 2, 6, 7, 16, 19, 20, 32, 53, 54, 55, 59, 68, 69, 74, 75, 84, 85, 104, 105, 106, 107, and the reference strain ATCC, as well the MRSA collection.

With the aim of reinforcing the results of the molecular identification, the identification of 20 isolated was performed using the MALDI-TOF MS. In this study, five bacterial species (*Bacillus pumilus* - isolate 6; *S. warneri* - isolates 7, 16, 68, 69, 74, 84, 85, 92, 19, and 53; *Micrococcus luteus* - isolates 31 and 91; *S. aureus* – isolates 75, 106, and 107; and *S. saprophyticus* – isolates 97, 98, 104, and 105) were identified. From the 20 isolates tested, only three did not belong to family of Staphylococcaceae. Table 3.1.2 presents the comparison between 16S sequences and MALDI-TOF MS. The comparison revealed differences in the identification of isolates 6 and 91. Isolate 6 was identified as *S. aureus* using 16S and positive for Sa, but with MALDI-TOF MS was identified as *Bacillus pumilus.* Isolate 91 was identified as *S. warneri* while with MALDI-TOF MS was identified as *M. luteus*. One explanation for this discrepancy might be that the primer is not as specific as mentioned, making uncertain results,

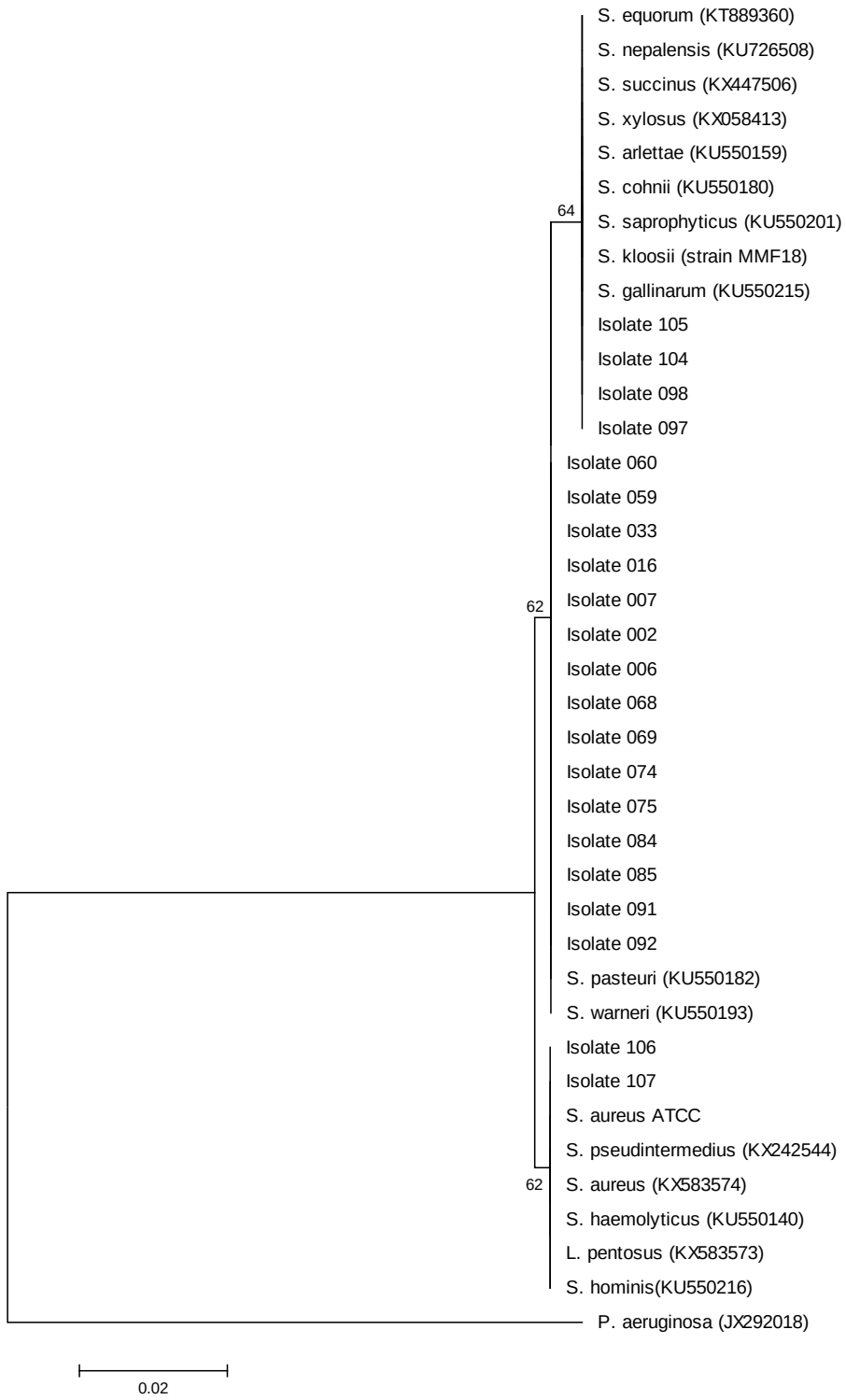


Figure 3.1.1 - Molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model [61]. The tree with the highest log likelihood (0.0000) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 39 nucleotide sequences. There were a total of 237 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [55].

since the MALDI-TOF MS is more reliable than the identification using Sa primers. Recently, some studies compared the performance of the MALDI-TOF MS identification in routine processes, such as the study by Bizzini and Greub [62]. In this study, some differences in bacterial identification were reported, but the MALDI-TOF MS process was the preferred for authors, although it has the necessity to require to 16S rRNA sequencing in few cases for which no reference spectra present in the MALDI-TOF MS databases at the time of analysis [62]. Many authors advocated that this technology is more accurate than other conventional identifying tests. For example, Charretier and co-workers [63] highlighted that the MALDI-TOF MS had to become “the new gold standard for bacterial identification”. Regarding the results of the 16S sequence, these results presented more doubts, due to the high E-value obtained. However, the average score obtained in the MALDI-TOF MS presented acceptable values.

In the literature, some authors showed new applications of the MALDI-TOF MS process, such as the microbiological diagnostic and resistant microorganism detection [64]. As it depends on modifications in the cell wall and proteome-related structural alterations, some studies have shown the capacity of MALDI-TOF MS to distinguish a susceptible bacteria from a resistant bacteria, for example, the methicillin resistance in *S. aureus* strains [62]. A possible next step for this thesis would be the detection of bacterial resistances using this process.

Table 3.1.2 - Sequencing and MALDI-TOF results for the bacterial isolates included in the present study.

Isolates	16S							MALDI-TOF	
	Description	**Max Score**	**Total Score**	**Query cover**	**E-value**	**Identity**	**Accession**	**Organism**	**Average Score**
2	*S. aureus*	454	454	100%	$2E^{-124}$	100%	KR025521		
6	*S. aureus*	454	454	100%	$2E^{-124}$	100%	KR025521	*Bacillus pumilus*	1,841
7	*S. warneri*	454	454	100%	$2E^{-124}$	100%	KU550193	*S. warneri*	1,919
16	*S. warneri*	454	454	100%	$2E^{-124}$	100%	KU550193	*S. warneri*	1,743
19								*S. warneri*	1,912
31								*Micrococcus luteus*	1,537
33	*S. warneri*	454	454	100%	$2E^{-124}$	100%	KU550193		
53								*S. warneri*	1,749
59	*S. aureus*	454	454	100%	$2E^{-124}$	100%	KR025521		
60	*S. warneri*	454	454	100%	$2E^{-124}$	100%	KU550193		
68	*S. warneri*	454	454	100%	$2E^{-124}$	100%	KU550193	*S. warneri*	1,776
69	*S. warneri*	454	454	100%	$2E^{-124}$	100%	KU550193	*S. warneri*	1,909
74	*S. warneri*	454	454	100%	$2E^{-124}$	100%	KU550193	*S. warneri*	1,782
75	*S. aureus*	454	454	100%	$2E^{-124}$	100%	KR025521	*S. aureus*	2,286
84	*S. warneri*	454	454	100%	$2E^{-124}$	100%	KU550193	*S. warneri*	1,963
85	*S. warneri*	454	454	100%	$2E^{-124}$	100%	KU550193	*S. warneri*	1,895
91	*S. warneri*	454	454	100%	$2E^{-124}$	100%	KU550193	*Micrococcus luteus*	1,825
92	*S. warneri*	454	454	100%	$2E^{-124}$	100%	KU550193	*S. warneri*	1,848
97	*S. saprophyticus*	454	454	100%	$2E^{-124}$	100%	KU550201	*S. saprophyticus*	2,003
98	*S. saprophyticus*	454	454	100%	$2E^{-124}$	100%	KU550201	*S. saprophyticus*	1,804
104	*S. saprophyticus*	454	454	100%	$2E^{-124}$	100%	KU550201	*S. saprophyticus*	1,792
105	*S. saprophyticus*	454	454	100%	$2E^{-124}$	100%	KU550201	*S. saprophyticus*	2,033
106	*S. aureus*	454	3635	100%	$2E^{-124}$	100%	KX583574	*S. aureus*	2,260
107	*S. aureus*	454	3635	100%	$2E^{-124}$	100%	KX583574	*S. aureus*	2,338
ATCC	*S. aureus*	454	3635	100%	$2E^{-124}$	100%	KX583574		

3.2 Antibiotic resistance detection

3.2.1 Molecular detection of antibiotic resistance

In the table 3.1.1 are presented the results of amplification with the primers associated to antibiotic resistances (*aacA-aphD*, *mecA*, *vanA*, *vanB*, *vanC*, and *qacC*).

The *aacA-aphD* gene has been used for the identification of aminoglycoside resistance [51]. Among the 30 bacterial isolates analyzed, three (isolates 68, 74, and 75) presented this gene. On other hand, the gene *aacA-aphD* was present in all the isolates reported as multi-resistant strains. In previous works by Adwan and co-workers [65], the same primer set was used to assess the resistance to aminoglycoside in methicillin-resistant bacteria. The presence of the *aacA-aphD* gene enables the resistance to aminoglycosides, such as macrolides, lincosamides and streptogramin (MLS group), used in the treatment of staphylococcal infections. This resistance is modulated by reduced uptake, production of aminoglycoside modifying enzymes and decreased cell permeability [65].

The *mecA* gene has been used for the identification of methicillin resistance. Among the 30 isolates analyzed, just one sample was positive for the presence of this gene. This assay was performed as described by Strommenger and coworkers [51], that indicated a fragment size of 532bp. However, the size of the amplified fragment was proximally 300bp. Faria and co-workers [66] used the same primers, obtaining fragments with different sizes, 314bp. Therefore, the results from the MRSA collection amplification can be considered as positive for methicillin resistance. The amplification of sample 92 originated a fragment size with proximally 300bp, also considered as positive. In work developed by Volkmann and coworkers [67], for detection of clinically relevant antibiotic resistance genes in municipal wastewater, the *mecA* gene was not found in municipal wastewater samples. However, the presence of this gene was detected in clinical wastewater samples [67].

In work developed by Zhang and coworkers [68], vancomycin resistance genes were detected in environmental waters, being *vanA* and *vanB* the most frequent genes associated with this resistance. Such as confirmed by Ateba and coworkers [69], *vanA* and *vanB* genes were found in groundwater. In this study, the VRE strain was the only one that presented positive results to vancomycin resistance (*vanA* gene), as was expected. The remaining isolates presented negative results. The PCR process with other vancomycin genes, *vanB* and *vanC*, also presented negative results for all

isolates. None of the isolates amplified the vancomycin-resistance gene, being all isolates susceptible to vancomycin, except the VRE strain. Similar results were obtained by Miele and coworkers [70], where they tested the specificity of each primer (*vanA*, *vanB*, and *vanC*). The group demonstrated that the region of the gene that is amplified by *vanA* can be amplified by other primers, while the region of the gene that is amplified by *vanB* or *vanC* can only be amplified by the respective pair primers (*vanB* and *vanC*).

The *qacC* gene has been used for the identification disinfectant resistance Among the 30 isolates analyzed, none was successfully amplified, suggesting that all the analyzed isolates were susceptible to this disinfectant commonly used in the water treatment. Contrarily, the work performed by Sekiguchi and coworkers [53], using the same method and primer, obtained positive results in hospital wastewater.

Several studies report antibiotic resistance in wastewater, such as the study by Borjesson and coworkers [71], which used the antibiogram to identify the resistant bacteria in municipal wastewater.

In table 3.2.1.1 are presented the results of the antibiograms performed in this study. All the analyzed isolates were susceptible to moxifloxacin, tetracycline, gentamicin, cefuroxime, levofloxacin, cefoxitin, ciprofloxacin, imipenem, rifampicin, and linezolid. Five isolates (53, 69, 75, 91, and 106) were susceptible to all tested antibiotics, one sample (isolate 68) was resistant to one antibiotic, and 14 isolates were resistant to at least two antibiotics (two antibiotics: isolate 107; three antibiotics: isolates 60, 74, 84, 85, and 92; four antibiotics: isolates 7 and 16; five antibiotics: isolate 105; six antibiotics: isolates 59 and 104; nine antibiotics: isolates 97 and 98; average resistance: four antibiotics). The most common the simultaneous resistance pattern was erythromycin and clindamycin (n = 8, 42.1%) followed by penicillin, ceftriaxone, cefotaxime and fosfomycin (n = 4, 28.6%). Among the analyzed samples, fourteen (73.7%) were resistant to fusidic acid. Fusidic acid, a bacteriostatic antibiotic [72], is frequently used against coagulase- negative and positive Staphylococci, Corynebacteria, many strict anaerobes, and Microaerophiles [72]. The increase of bacteria resistant to this antibiotic was been previously reported by Fijałkowski and coworkers [73] and Gòmez and coworkers [74] and is associated with mutations in five genes – *fusA*, *fusB*, *fusc*, *fusD*, *fusE* [75]. It has been suggested that the emergence of fusidic acid-resistances may be attributed to its widespread clinical use, mainly in combination with other antibiotics to combat severe infections. Eight isolates (42.1%) were simultaneously resistant to both erythromycin and clindamycin. Although

belonging to different antimicrobial classes, erythromycin - a macrolide- and clindamycin - a lincosamide, these two substances disrupt bacterial cells acting on ribosomal subunits [76]. This combined resistance has been previously described in Staphylococci [77] and Streptococci [78]. The combined used of these antibiotics may have contributed to the occurrence of this multi-resistance pattern. Four isolates were simultaneously resistant to penicillin, ceftriaxone, cefotaxime and fosfomycin. Fosfomycin presents a broad-spectrum activity against gram-negative positive bacteria [79]. This antibiotic can be used alone, in cases of mild infections [79], or in combination with a cephalosporin (such as ceftriaxone and cefotaxime) and β-lactam antibiotics (such as penicillin), in more severe infections [80]. As mentioned in the earlier case, the combined antibiotic prescription may have contributed to this multi-resistance pattern. Therefore, 63.2% of the samples presented resistance to at least three antibiotics, and according to Onanuga and coworkers [81], and Poma and coworkers [82] these are defined multi-resistant bacteria. The both works are related the occurrence of multi-resistant antibiotic in patients with urinary tract infections and enteric pathogens in river water, irrigated soil and fresh vegetables, respectively. So, 63.2 % (n=12) of the samples analyzed in this thesis are multi-resistant (namely the isolates 7, 16, 59, 60, 74, 84, 85, 92, 97, 98, 104 and 105).

Table 3.2.1.1 - Antibiogram results (Legend: P1 – penicillin1; MXF 5 – moxifloxacin 5; E15 – erythromycin 15; CC2 – clindamycin 2; CN30 – cephalexin; TE30 – tetracyclin 30; SXT – cotrimazol 25; AM10 – ampicillin 10; GM10 – gentamicin 10; CXM30 – cefuroxin 30; LVX5 – levofloxacin 5; FOX30 – cefoxitin 30; CIP5 – ciprofloxacin 5; TZP36 – tazonam 36; CRO 30 – ceftriaxone 30; CTX5 – cefotaxime 5; IPM10 – imipenem 10; FOS 200 – fofomycin 200; RA5 – rifampicin 5; FA10 – fusidic acid 10; LZD10 – linezolid 10. (s) – susceptible. (ns) – non susceptible).

	Antibiogram																				
Samples	**P 1**	**MXF 5**	**E 15**	**CC 2**	**CN 30**	**TE 30**	**SXT**	**AM 10**	**GM 10**	**CXM 30**	**LVX 5**	**FOX 30**	**CIP 5**	**TZP 36**	**CRO 30**	**CTX 5**	**IPM 10**	**FOS 200**	**RA 5**	**FA 10**	**LZD 10**
7	s	s	ns	ns	s	s	s	s	s	s	s	s	s	s	s	s	s	ns	s	ns	s
16	s	s	ns	ns	s	s	s	s	s	s	s	s	s	s	s	s	s	ns	s	ns	s
53	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s
59	ns	s	ns	ns	ns	s	s	s	s	s	s	s	s	s	s	s	s	ns	s	ns	s
60	s	s	ns	ns	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	ns	s
68	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	ns	s
69	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s
74	s	s	ns	ns	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	ns	s
75	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s
84	s	s	ns	ns	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	ns	s
85	s	s	ns	ns	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	ns	s
91	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s
92	s	s	ns	ns	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	ns	s
97	ns	s	s	s	ns	s	ns	ns	s	s	s	s	s	ns	ns	ns	s	ns	s	ns	s
98	ns	s	s	s	ns	s	ns	ns	s	s	s	s	s	ns	ns	ns	s	ns	s	ns	s
104	ns	s	s	s	ns	s	s	s	s	s	s	s	s	s	ns	ns	s	ns	s	ns	s
105	ns	s	s	s	s	s	s	s	s	s	s	s	s	s	ns	ns	s	ns	s	ns	s
106	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s	s
107	s	s	s	s	ns	s	s	s	s	s	s	s	s	s	s	s	s	s	s	ns	s

4. Conclusion

Nowadays, antibiotic resistance is an important public health concern. The use and misuse of antibiotics in humans, veterinary, and agricultural have contributed to the increase of resistant microorganisms. Additionally, the HGT has enabled the high spread of resistance between bacteria, as well as mutations.

The presence of antibiotic resistant microorganisms in environmental waters has augmented too, which was associated with the incorrect discharging of antibiotics wastes. The presence of antibiotics in wastewater enables the proliferation of antibiotic resistance among bacteria.

In this work, the majority of samples were identified as *Staphylococcus* species, which presented a significant capacity to acquire antibiotic-resistance, such as the multi-resistant *S. aureus*. Through molecular phylogenetic analysis, was possible to identify the relationship between the samples and the others strains, such as *S. warneri*, *S. aureus* and *S. saprophyticus*.

The molecular identification of resistances with the tested resistant genes enabled the observation of resistant samples. However, only a few isolates amplified the studied genes, thus showing their resistance to antibiotics.

The antibiogram results demonstrated that a high number of bacteria is resistant to fusidic acid, while showing that all samples presented a high susceptibility to other antibiotics, such as moxifloxacin. Therefore, the majority of the samples showed resistance to at least one antibiotic.

In conclusion, the spread of antibiotic resistance in environmental waters is an actual concern to public health and is necessary the reduction of undue consumption of antibiotics and their spread for environment. Furthermore, there is a need for new techniques to significantly reduce or eliminate the microorganisms resistant to antibiotics in the environment.

References

1. Rossolini, G.M., et al., *Update on the antibiotic resistance crisis.* Current Opinion in Pharmacology, 2014. **18**: p. 56-60.

2. Baquero, F., A.-S.P. Tedim, and T.M. Coque, *Antibiotic resistance shaping multilevel population biology of bacteria.* Frontiers in Microbiology, 2013. **4**.

3. Ferri, M., et al., *Antimicrobial Resistance: A Global Emerging Threat to Public Health Systems.* Critical Reviews in Food Science and Nutrition, 2015.

4. Li, W.C., *Occurrence, sources, and fate of pharmaceuticals in aquatic environment and soil.* Environmental Pollution, 2014. **187**: p. 193-201.

5. Nathan, C. and O. Cars, *Antibiotic Resistance — Problems, Progress, and Prospects.* New England Journal of Medicine, 2014. **371**(19): p. 1761-1763.

6. Wright, G.D. and H. Poinar, *Antibiotic resistance is ancient: implications for drug discovery.* Trends in Microbiology, 2012. **20**(4): p. 157-159.

7. Laxminarayan, R., et al., *Antibiotic resistance—the need for global solutions.* The Lancet Infectious Diseases, 2013. **13**(12): p. 1057-1098.

8. Lin, J., et al., *Mechanisms of antibiotic resistance.* Frontiers in Microbiology, 2015. **6**.

9. Davies, J. and D. Davies, *Origins and Evolution of Antibiotic Resistance.* Microbiology and Molecular Biology Reviews, 2010. **74**(3): p. 417-433.

10. Livermore, D.M., *Minimising antibiotic resistance.* The Lancet Infectious Diseases, 2005. **5**(7): p. 450-459.

11. ECDC, *Antimicrobial resistancesurveillance in Europe - Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2014*. 2015, European Union and Publications Office. p. 130.

12. Rodríguez-Rojas, A., et al., *Antibiotics and antibiotic resistance: A bitter fight against evolution.* International Journal of Medical Microbiology, 2013. **303**(6–7): p. 293-297.

13. Neu, H.C., *The Crisis in Antibiotic Resistance.* Science, 1992. **257**(5073): p. 1064-1073.

14. Ventola, C.L., *The Antibiotic Resistance Crisis: Part 1: Causes and Threats.* Pharmacy and Therapeutics, 2015. **40**(4): p. 277-283.

15. da Costa, P.M., L. Loureiro, and A.J. Matos, *Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals and the environment.* Int J Environ Res Public Health, 2013. **10**(1): p. 278-94.

16. Argudín, M.A., et al., *Molecular basis of antimicrobial drug resistance in Staphylococcus aureus isolates recovered from young healthy carriers in Spain.* Microbial Pathogenesis, 2014. **74**: p. 8-14.

17. Ambretti, S., et al., *Reporting epidemiology of antibiotic resistance.* 2015, 2015. **30**(2).

18. Andersson, D.I., *Evolution of antibiotic resistance*, in *The Princeton Guide to Evolution*. 2013, Princeton University Press. p. 770-776.

19. Martinez, J.L., *The role of natural environments in the evolution of resistance traits in pathogenic bacteria.* Proceedings of the Royal Society of London B: Biological Sciences, 2009. **276**(1667): p. 2521-2530.

20. Gordo, I., L. Perfeito, and A. Sousa, *Fitness Effects of Mutations in Bacteria.* Journal of Molecular Microbiology and Biotechnology, 2011. **21**(1-2): p. 20-35.

21. Andersson, D.I. and D. Hughes, *Antibiotic resistance and its cost: is it possible to reverse resistance?* Nat Rev Micro, 2010. **8**(4): p. 260-271.

22. Smith, T.L. and W.R. Jarvis, *Antimicrobial resistance in Staphylococcus aureus.* Microbes and Infection, 1999. **1**(10): p. 795-805.

23. Jain, R., et al., *Horizontal Gene Transfer in Microbial Genome Evolution.* Theoretical Population Biology, 2002. **61**(4): p. 489-495.

24. Philippe, H. and C.J. Douady, *Horizontal gene transfer and phylogenetics.* Current Opinion in Microbiology, 2003. **6**(5): p. 498-505.

25. Cox, G. and G.D. Wright, *Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions.* International Journal of Medical Microbiology, 2013. **303**(6–7): p. 287-292.

26. Juhas, M., *Horizontal gene transfer in human pathogens.* Critical Reviews in Microbiology, 2015. **41**(1): p. 101-108.

27. van Hoek, A.H.A.M., et al., *Acquired antibiotic resistance genes: an overview.* Frontiers in Microbiology, 2011. **2**.

28. de la Cruz, F. and J. Davies, *Horizontal gene transfer and the origin of species: lessons from bacteria.* Trends in Microbiology, 2000. **8**(3): p. 128-133.

29. Shin, J.-E., C. Lin, and H.N. Lim, *Horizontal transfer of DNA methylation patterns into bacterial chromosomes.* Nucleic Acids Research, 2016. **44**(9): p. 4460-4471.

30. Burmeister, A.R., *Horizontal Gene Transfer.* Evolution, Medicine, and Public Health, 2015. **2015**(1): p. 193-194.

31. Babić, A., et al., *Direct Visualization of Horizontal Gene Transfer.* Science, 2008. **319**(5869): p. 1533-1536.

32. Ragan, M.A., *Detection of lateral gene transfer among microbial genomes.* Current Opinion in Genetics & Development, 2001. **11**(6): p. 620-626.

33. Malachowa, N. and F.R. DeLeo, *Mobile genetic elements of Staphylococcus aureus.* Cellular and Molecular Life Sciences, 2010. **67**(18): p. 3057-3071.

34. Syvanen, M. and C.I. Kado, *Horizontal Gene Transfer*. 2001: Elsevier Science.

35. Syvanen, M., *Evolutionary Implications of Horizontal Gene Transfer.* Annual Review of Genetics, 2012. **46**(1): p. 341-358.

36. Krüger, N.-J. and K. Stingl, *Two steps away from novelty – principles of bacterial DNA uptake.* Molecular Microbiology, 2011. **80**(4): p. 860-867.

37. Juhas, M., *Type IV secretion systems and genomic islands-mediated horizontal gene transfer in Pseudomonas and Haemophilus.* Microbiological research, 2015. **170**: p. 10-17.

38. Harrison, E. and M.A. Brockhurst, *Plasmid-mediated horizontal gene transfer is a coevolutionary process.* Trends in Microbiology, 2012. **20**(6): p. 262-267.

39. Brown-Jaque, M., W. Calero-Cáceres, and M. Muniesa, *Transfer of antibiotic-resistance genes via phage-related mobile elements.* Plasmid, 2015. **79**: p. 1-7.

40. Huddleston, J.R., *Horizontal gene transfer in the human gastrointestinal tract: Potential spread of antibiotic resistance genes.* Infect. Drug Resist, 2014. **7**: p. 167-176.

41. Zhang, Y., et al., *Microbial community functional structure in response to antibiotics in pharmaceutical wastewater treatment systems.* Water Research, 2013. **47**(16): p. 6298-6308.

42. Rizzo, L., et al., *Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review.* Science of The Total Environment, 2013. **447**: p. 345-360.

43. Taylor, D. and T. Senac, *Human pharmaceutical products in the environment – The "problem" in perspective.* Chemosphere, 2014. **115**: p. 95-99.

44. Huerta, B., et al., *Exploring the links between antibiotic occurrence, antibiotic resistance, and bacterial communities in water supply reservoirs.* Science of The Total Environment, 2013. **456–457**: p. 161-170.

45. Baquero, F., J.-L. Martínez, and R. Cantón, *Antibiotics and antibiotic resistance in water environments.* Current Opinion in Biotechnology, 2008. **19**(3): p. 260-265.

46. Martins da Costa, P., P. Vaz-Pires, and F. Bernardo, *Antimicrobial resistance in Enterococcus spp. isolated in inflow, effluent and sludge from municipal sewage water treatment plants.* Water Research, 2006. **40**(8): p. 1735-1740.

47. Masoud, G., et al., *Bacteriological quality of some swimming pools in Alexandria with special reference to Staphylococcus aureus.* Environmental Monitoring and Assessment, 2016. **188**(7): p. 1-8.

48. Qu, L., et al., *Efficient and sensitive method of DNA silver staining in polyacrylamide gels.* ELECTROPHORESIS, 2005. **26**(1): p. 99-101.

49. Martineau, F., et al., *Species-specific and ubiquitous DNA-based assays for rapid identification of Staphylococcus epidermidis.* Journal of Clinical Microbiology, 1996. **34**(12): p. 2888-93.

50. Martineau, F., et al., *Species-Specific and Ubiquitous-DNA-Based Assays for Rapid Identification of Staphylococcus aureus.* Journal of Clinical Microbiology, 1998. **36**(3): p. 618-623.

51. Strommenger, B., et al., *Multiplex PCR Assay for Simultaneous Detection of Nine Clinically Relevant Antibiotic Resistance Genes in Staphylococcus aureus.* Journal of Clinical Microbiology, 2003. **41**(9): p. 4089-4094.

52. Clark, N.C., et al., *Characterization of glycopeptide-resistant enterococci from U.S. hospitals.* Antimicrobial Agents and Chemotherapy, 1993. **37**(11): p. 2311-2317.

53. Sekiguchi, J., et al., *Detection of the antiseptic- and disinfectant-resistance genes qacA, qacB, and qacC in methicillin-resistant Staphylococcus aureus isolated in a Tokyo hospital.* Jpn J Infect Dis, 2004. **57**(6): p. 288-91.

54. Kearse, M., et al., *Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data.* Bioinformatics, 2012. **28**(12): p. 1647-1649.

55. Tamura, K., et al., *MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0.* Molecular Biology and Evolution, 2013. **30**(12): p. 2725-2729.

56. Stevenson, L.G., et al., *Evaluation of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for Identification of Clinically Important Yeast Species.* Journal of Clinical Microbiology, 2010. **48**(10): p. 3482-3486.

57. Matuschek, E., D.F.J. Brown, and G. Kahlmeter, *Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories.* Clinical Microbiology and Infection, 2014. **20**(4): p. O255-O266.

58. Ivone, V.-M., et al., *Bacterial diversity from the source to the tap: a comparative study based on 16S rRNA gene-DGGE and culture-dependent methods.* FEMS Microbiology Ecology, 2013. **83**(2): p. 361-374.

59. Farkas, A., et al., *Microbiological contamination and resistance genes in biofilms occurring during the drinking water treatment process.* Science of The Total Environment, 2013. **443**: p. 932-938.

60. Pereira, E.M., et al., *Staphylococcus aureus, Staphylococcus epidermidis and Staphylococcus haemolyticus: Methicillin-resistant isolates are detected directly in blood cultures by multiplex PCR.* Microbiological Research, 2010. **165**(3): p. 243-249.

61. Jukes, T.H. and C.R. Cantor, *Evolution of protein molecules.* Mammalian protein metabolism. **3**(21): p. 132.

62. Bizzini, A. and G. Greub, *Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification.* Clinical Microbiology and Infection, 2010. **16**(11): p. 1614-1619.

63. Charretier, Y., et al., *Rapid Bacterial Identification, Resistance, Virulence and Type Profiling using Selected Reaction Monitoring Mass Spectrometry.* Scientific Reports, 2015. **5**: p. 13944.

64. De Carolis, E., et al., *Application of MALDI-TOF mass spectrometry in clinical diagnostic microbiology*. 2014. Vol. 8. 2014.

65. Adwan, G., et al., *Molecular detection of nine antibiotic resistance genes in methicillin resistant Staphylococcus aureus isolates.* Roum Arch Microbiol Immunol, 2014. **73**(1-2): p. 9-17.

66. Faria, C., et al., *Antibiotic resistance in coagulase negative staphylococci isolated from wastewater and drinking water.* Science of The Total Environment, 2009. **407**(12): p. 3876-3882.

67. Volkmann, H., et al., *Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan).* Journal of Microbiological Methods, 2004. **56**(2): p. 277-286.

68. Zhang, X.-X., T. Zhang, and H.H.P. Fang, *Antibiotic resistance genes in water environment.* Applied Microbiology and Biotechnology, 2009. **82**(3): p. 397-414.

69. Ateba, C.N., K.P. Lekoma, and D.T. Kawadza, *Detection of vanA and vanB genes in vancomycin-resistant enterococci (VRE) from groundwater using multiplex PCR analysis.* J Water Health, 2013. **11**(4): p. 684-91.

70. Miele, A., M. Bandera, and B.P. Goldstein, *Use of primers selective for vancomycin resistance genes to determine van genotype in enterococci and to study gene organization in VanA isolates.* Antimicrobial Agents and Chemotherapy, 1995. **39**(8): p. 1772-1778.

71. Borjesson, S., et al., *Methicillin-resistant Staphylococcus aureus (MRSA) in municipal wastewater: an uncharted threat?* J Appl Microbiol, 2010. **108**(4): p. 1244-51.

72. Verbist, L., *The antimicrobial activity of fusidic acid.* Journal of Antimicrobial Chemotherapy, 1990. **25**(suppl B): p. 1-5.

73. Fijałkowski, K., D. Peitler, and J. Karakulska, *Staphylococci isolated from ready-to-eat meat – Identification, antibiotic resistance and toxin gene profile.* International Journal of Food Microbiology, 2016. **238**: p. 113-120.

74. Gómez, P., et al., *Characterization of staphylococci in urban wastewater treatment plants in Spain, with detection of methicillin resistant Staphylococcus aureus ST398.* Environmental Pollution, 2016. **212**: p. 71-76.

75. den Heijer, C.D.J., et al., *Fusidic acid resistance in Staphylococcus aureus nasal carriage strains in nine European countries.* Future Microbiology, 2014. **9**(6): p. 737-745.

76. Levin, T.P., et al., *Potential Clindamycin Resistance in Clindamycin-Susceptible, Erythromycin-Resistant Staphylococcus aureus: Report of a Clinical Failure.* Antimicrobial Agents and Chemotherapy, 2005. **49**(3): p. 1222-1224.

77. Fiebelkorn, K.R., et al., *Practical Disk Diffusion Method for Detection of Inducible Clindamycin Resistance in Staphylococcus aureus and Coagulase-Negative Staphylococci.* Journal of Clinical Microbiology, 2003. **41**(10): p. 4740-4744.

78. Gygax, S.E., et al., *Erythromycin and Clindamycin Resistance in Group B Streptococcal Clinical Isolates.* Antimicrobial Agents and Chemotherapy, 2006. **50**(5): p. 1875-1877.

79. Falagas, M.E., et al., *Fosfomycin: Use Beyond Urinary Tract and Gastrointestinal Infections.* Clinical Infectious Diseases, 2008. **46**(7): p. 1069-1077.

80. Chavanet, P., et al., *In vivo activity and pharmacodynamics of cefotaxime or ceftriaxone in combination with fosfomycin in fibrin clots infected with highly penicillin-resistant Streptococcus pneumoniae.* Antimicrobial Agents and Chemotherapy, 1995. **39**(8): p. 1736-43.

81. Onanuga, A. and G.O. Awhowho, *Antimicrobial resistance of Staphylococcus aureus strains from patients with urinary tract infections in Yenagoa, Nigeria.* Journal of Pharmacy & Bioallied Sciences, 2012. **4**(3): p. 226-230.

82. Poma, V., N. Mamani, and V. Iñiguez, *Impact of urban contamination of the La Paz River basin on thermotolerant coliform density and occurrence of multiple antibiotic resistant enteric pathogens in river water, irrigated soil and fresh vegetables.* SpringerPlus, 2016. **5**(1): p. 1-11.

Appendix

Appendix 1

Table of samples used in this thesis.

Sample	Isolation date	Petri dish code	Colony color
2	2016.01.26	#2_8382	White
6			
7			
16			
17			Yellow
18			
19			
20		#1_8281	White
31			
32			Yellow
33			
53			White
54		#2_8380	Yellow
55			
59	2016.04.28	#7_20_A	White
60			
68			Yellow
69			
74		#7_20_B	White
75			
84			Yellow
85			
91		#7_20_C	White
92			
97		#8_20_B	Yellow
98			
104			Pink

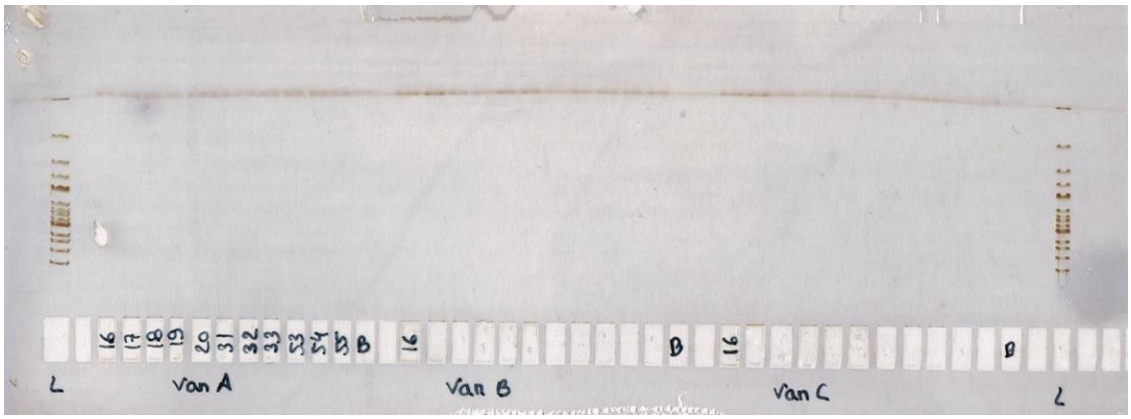
105			
106	2016.04.28	45_19	Yellow
107			
ATCC	-	-	FFUP
VRE			
110			MRSA (IMU)
111			
112			
113			
114			
115			
116			
117			
119			
120			

Appendix 2

Amplification results of the *16S, aacA-aphD, mecA*, and *qacC* genes.

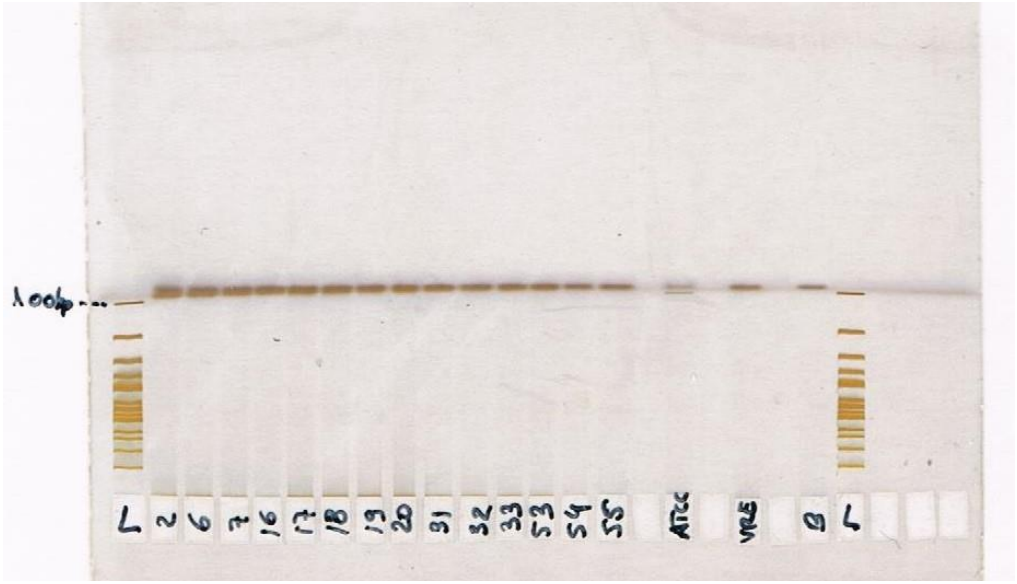
Appendix 3

Amplification results of the *vanA, vanB* and *vanC* genes.



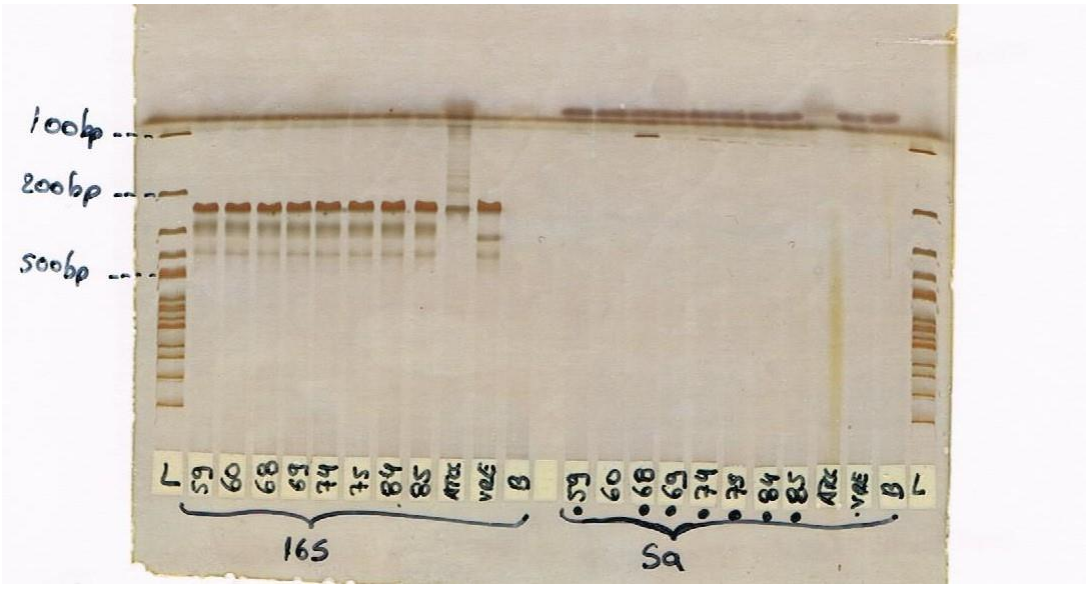
Appendix 4

Amplification results of the genomic region *Sa*.



Appendix 5

Amplification results of the *16S* and *Sa* genomic regions.

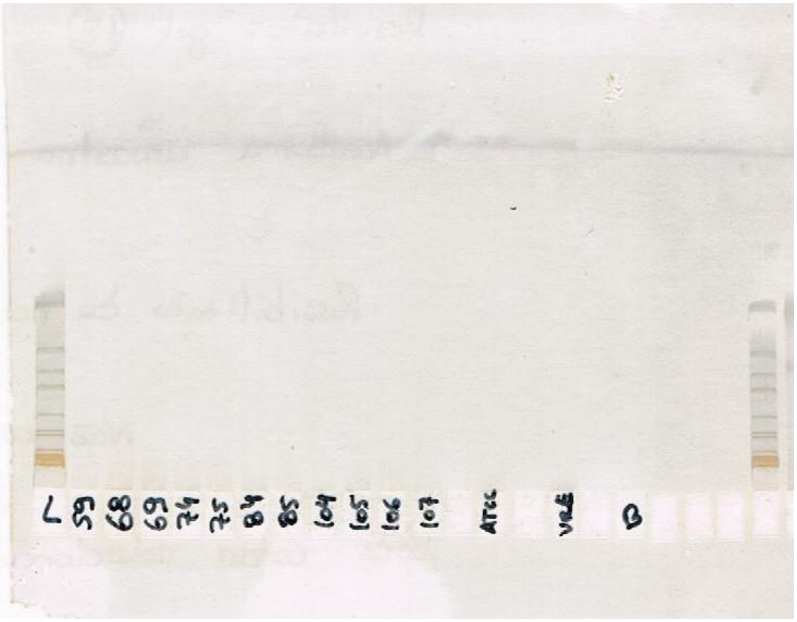


Appendix 6

Amplification results of the *aacA-aphD* gene.

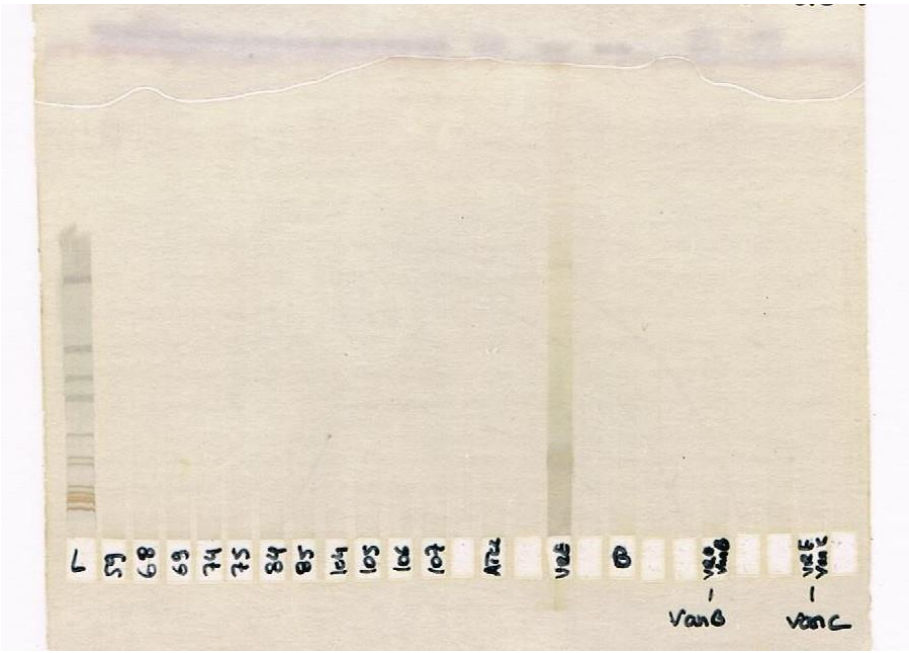
Appendix 7

Amplification results of the *qacC* gene.



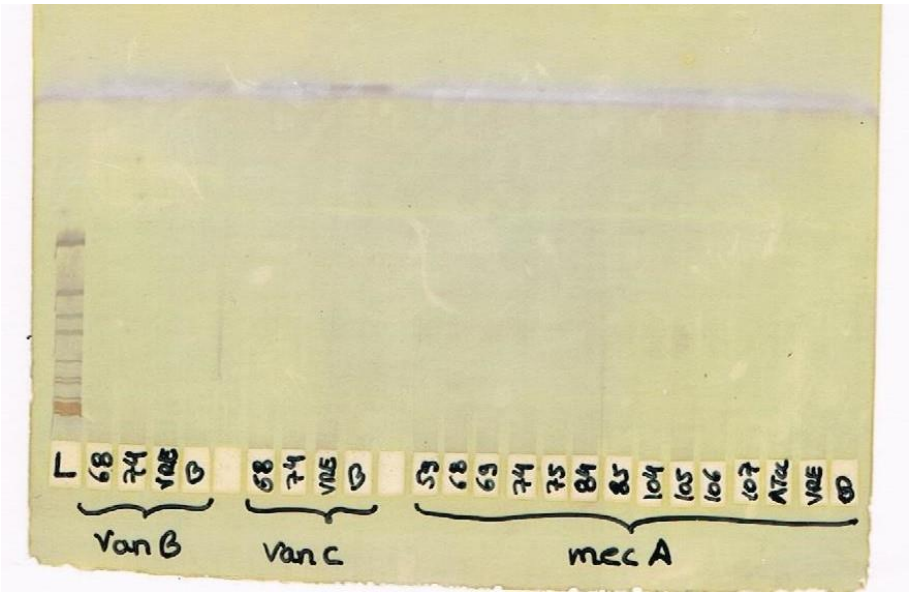
Appendix 8

Amplification results of the *vanA* gene.



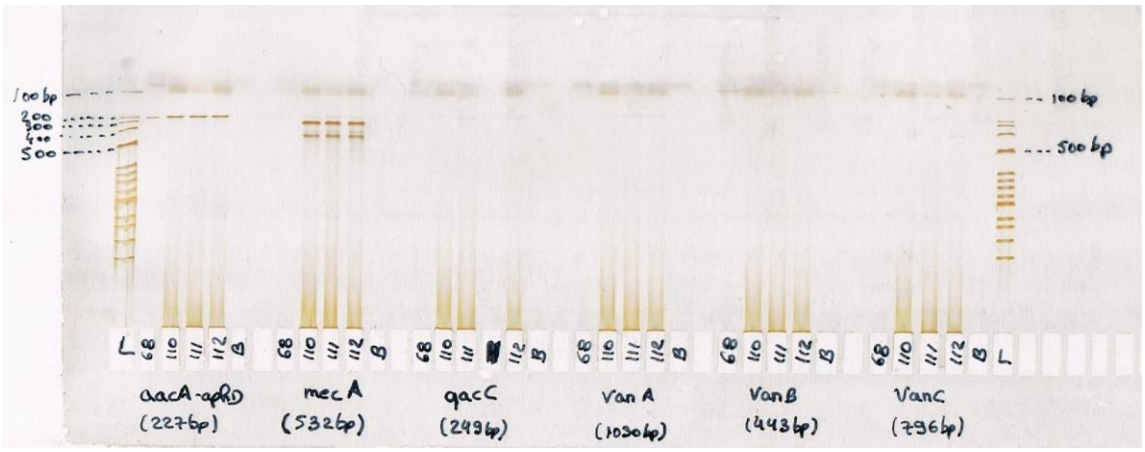
Appendix 9

Amplification results of the *vanB, vanC* and *mecA* genes.



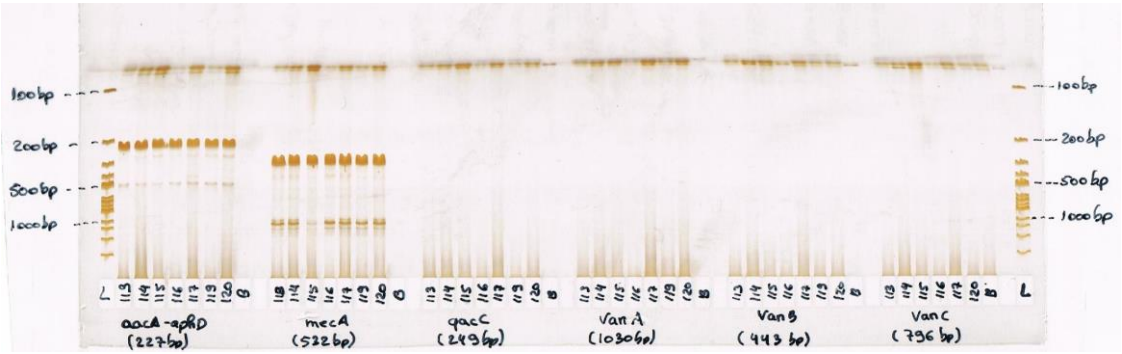
Appendix 10

Amplification results of the *aacA-aphD, mecA, qacC, vanA, vanB* and *vanC* genes.



Appendix 11

Amplification results of the *aacA-aphD, mecA, qacC, vanA, vanB* and *vanC* genes.



Appendix 12

Amplification results of the *aacA-aphD, mecA, qacC, vanA, vanB* and *vanC* genes.